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Dependence of Thiol-Stimulated K-Cl Cotransport on Redox State of Low K Sheep Red Blood Cells

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109. Creatine Kinase Is Required for Swelling-activated K-Cl Cotransport in Dog Red Blood Cells G. CRAIG COLCLASURE, JOHN C. PARKER, and PHILIP B. DUNHAM, *Department of Medicine, University of North Carolina, Chapel Hill, North Carolina*

Volume-sensitive K-Cl cotransport in resealed dog red cell ghosts requires the incorporation of phosphocreatine before resealing, while the incorporation of ATP has no effect (Colclasure and Parker, 1993. *American Journal of Physiology*. 265:C1648). We explored the possibility that creatine kinase (CK) plays an important role in swelling-induced K-Cl cotransport. Dinitrofluorobenzene (DNFB), a covalent inhibitor of CK, abolished nearly completely K-Cl cotransport in intact dog red cells and in resealed ghosts made from DNFB-treated cells. Incorporation of exogenous CK into ghosts made from DNFB-treated cells fully restored K-Cl cotransport. Therefore, DNFB acts by inhibiting CK and not the cotransporter itself. Though dog red cells possess the BB (brain-derived) isoenzyme of CK, both BB and MM (muscle-derived) isoenzymes of CK from rabbit fully restored cotransport. CK activity of the ghosts was demonstrated by electrophoresis in agarose gels. The complete inhibition of native CK by DNFB and the incorporation of active CK were demonstrated. The dose/response curve for CK and K-Cl cotransport was determined in a CK reconstitution experiment. We estimate that 550 molecules of rabbit CK (BB) per resealed ghost restored 50% of the control level of cotransport. Because phosphocreatine is the substrate for only one enzyme, CK, it is likely that CK is providing ATP to a site which is inaccessible to bulk cytoplasmic ATP. The exact nature of this site and its role in swelling-activated K-Cl cotransport are uncertain. However the results of this study establish an essential role for CK is this system. (Supported by NIH grants DK-11356 and DK-33640.)

110. Differences in the Regulation of K:Cl Cotransport by Changes in Cell Volume in Hemoglobin SS, CC, and AA Red Cells JOSE R. ROMERO, CHRISTINE LAWRENCE, RONALD L. NAGEL, MARY E. FABRY, and MITZY CANESSA, *Brigham and Women's Hospital, Boston, Massachusetts, and Albert Einstein College of Medicine, Bronx, New York*

Subjects homozygous for hemoglobin A (AA), C (CC) and S (SS) exhibit different red blood cell (RBC) volumes which might be related to differences in cell volume regulation. We have investigated how rapidly K:Cl cotransport is activated and deactivated to regulate the cell volume in these cells. We measured the time course of K^+ efflux after step changes in cell volume and determined the delay times (DT) for cotransport relaxation between the inactive (isotonic media) and active (hypotonic media) states. The DT for activation was very short in cells with a high percent reticulocytes: (SS, 10% retics, 1.7 ± 0.3 min delay, $n = 8$ subjects; AA, 10%, 4 ± 1 min, $n = 3$; CC, 11.6%, 4 ± 0.3, $n = 3$), and long in cells with low percent retics: (AA, 1.5%, 10 ± 1 min, $n = 8$; CC whole blood 6%, 10 ± 2 min, $n = 10$, $P < 0.02$ vs SS). The DTs for deactivation by cell shrinking were very short in SS (3 ± 0.4 min, $n = 8$, $P < 0.02$) and AA cells with high (2 ± 1, $n = 3$) and normal retics (2 ± 1 min, $n = 3$) but 8–15–fold longer in CC cells (29 ± 2 min, $n = 9$). Density fractionation of CC cells resulted in coenrichment of the top fraction in retics and in cotransport (fourfold) with short DT for activation (4 min) and long delay for deactivation (14 min). The DT for activation, but not for deactivation, increased markedly with increasing cell density thus indicating that all CC cells do not promptly shut off cotransport with cell shrinkage. In summary, (*a*) K:Cl cotransport is not only very active in young cells but it is also very rapidly activated and deactivated in young AA and SS cells by changes in cell volume. (*b*) DT for cotransport activation markedly increased with cell age and in mature cells with low cotransport rates, long DTs were observed. (*c*) The long delay for deactivation exhibited by even young CC cells induces a persistent loss of cellular K^+ after cell shrinkage which may contribute to the uniformly low cell volume, low K^+ and water content of CC cells.

111. Dependence of Thiol-stimulated K-Cl Cotransport on Redox State of Low K Sheep Red Blood Cells PETER K. LAUF, NORMA C. ADRAGNA, and NIHAL S. AGAR, *Departments of Physiology and Biophysics, and Pharmacology and Toxicology, Wright State*

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The thiol oxidant diamide (DM) stimulates K-Cl cotransport in low K (LK) sheep red blood cells once cellular glutathion (GSH) is oxidized to its dithiol GSSG. This effect is reversed after metabolic or chemical reconstitution of the GSH levels (Lauf, 1988. *Journal of Membrane Biology*. 101:179) suggesting redox involvement in the control of K-Cl cotransport. To further test this hypothesis we took two approaches. We first compared the dose responses of K-Cl cotransport activation and GSH oxidation/alkanethiolation by the reversible thiol reagents DM and methylmethane thiosulfonate (MMTS) and of GSH alkylation by the irreversible thiol reagent *N*-ethylmaleimide (NEM). Second, we studied the effect of elimination of GSH on K-Cl cotransport by conjugation to chlorodinitrobenzene (CDNB) via the intracellular GSH transferase prior to exposure to DM, NEM and MMTS. The absolute values of the slopes of K-Cl cotransport activation and of GSH oxidation or alkanethiolation were similar but of opposite sign for both DM and MMTS, and different from those for NEM, suggesting a functional relationship between the cellular GSH pool and K-Cl cotransport activity. In contrast, the concentration (mmol/liter cells) for 50% activation of K-Cl cotransport and 50% reduction of GSH were identical only for MMTS (1.4 vs 1.3) but different for DM (5.3 vs 1.7) and NEM (4.9 vs 2.5) suggesting differences in relative potencies to affect K-Cl cotransport and GSH levels. K-Cl cotransport increased significantly above base level only after > 90% GSH was depleted by CDNB (without reducing ATP). The net stimulation by CDNB was less than 20% of that achieved by DM whereas GSH thiols represent 15–20% of the total red cell thiol pool. Treatment with NEM, DM, and MMTS after CDNB failed to activate K-Cl cotransport to levels seen with these reagents alone suggesting that all three reagents affected the system via the GSH/GSSG redox system. Based on the dose response curves for NEM-induced K-Cl flux activation and GSH alkylation the mechanism by which NEM produces its effect appears to be different. The determinants of the thiol-activated K-Cl cotransporter may be either crucial thiol group(s) in the carrier molecule which become unavailable upon CDNB treatment, or oxidized GSH, GSSG, which acts through thiols in the regulatory moiety of the K-Cl cotransport complex. (Supported by NIH DK 37,160.)

112. Sidedness of H^+ Action on K-Cl Cotransport in DIDS pH_i-clamped Low K Sheep Red Blood Cells with Reduced Mg_i

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We have recently proposed a model for proton, H^+, and cellular magnesium, Mg_i, modulation of Cl-dependent K and Rb fluxes through the K-Cl cotransporter in volume-clamped low K (LK) sheep red blood cells (Lauf, Erdmann, and Adragna. 1994. *American Journal of Physiology*. 266:(*Cell Physiology*. 35):C95). We now have determined the sidedness for the H^+ action on K-Cl cotransport in these cells with total Mg_i reduced to 1/20 of control by the divalent cation ionophore A23187 and an external chelator. The HCO_3/Cl exchange inhibitor DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was used in combination with external sucrose to clamp cellular pH, pH_i, at values between 6.7 and 8.6, and cell volume close to that of normal cells. Ouabain-insensitive K efflux and Rb influx were then measured in Cl and NO_3 media at pH_o of 6–9 for each clamped pH_i and at various external Rb concentrations, $[Rb]_o$. The difference between the K(Rb) fluxes in Cl and NO_3 is the Cl-dependent K(Rb) flux component attributable to K-Cl cotransport. Our observations were: (*a*) DIDS blocked Cl and H^+ equilibration with the following pH_o effectiveness: 9.0 = 7.4 > 6.5. (*b*) Consistent with the reversible DIDS inhibition of K-Cl cotransport (Delpire and Lauf. 1992. *Journal of Membrane Biology*. 126:89), LK cells washed after DIDS treatment exhibited K-Cl cotransport activities similar to those of controls; however, DIDS stimulated significantly both K and Rb fluxes in NO_3, particularly at pH_o 9. (*c*) An increase in V_{max} of Cl-dependent Rb influx was observed in cells pH-clamped only at pH_i = 8.6 when pH_o was raised from 6.5 to 9. No major effect on K_m was seen. (*d*) The flux reversal points (mM $[Rb]_o$) determined by simultaneous measurements of Cl-dependent K efflux and Rb influx agreed with those estimated from the calculated ratio of the chemical driving forces. Both shifted from ~10 to 40 mM as intracellular Cl increased by about fourfold upon lowering pH_i from 8.6 to 6.7. We conclude that in low Mg_i LK sheep red blood cells, with pH_i clamped by DIDS, H^+ either reduce the